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ORIGINAL ARTICLE

Interaction with *Pantoea agglomerans* Modulates Growth and Melanization of *Sporothrix brasiliensis* and *Sporothrix schenckii*

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Abstract *Sporothrix brasiliensis* and *Sporothrix schenckii* stand as the most virulent agents of sporotrichosis, a worldwide-distributed subcutaneous mycosis. The origin of *Sporothrix* virulence seems to be associated with fungal interactions with organisms living in the same environment. To assess this hypothesis, the growth of these two species in association with *Pantoea agglomerans*, a bacterium with a habitat similar to *Sporothrix* spp., was evaluated. Growth, melanization, and gene expression of the fungus were compared in the presence or absence of the bacterium in the same culture medium. Both *S. brasiliensis* and *S. schenckii* grew in contact with *P. agglomerans* yielding heavily melanized conidia after 5 days of incubation at 30 °C in Sabouraud agar. This increased melanin production occurred around bacterial colonies, suggesting that fungal melanization is triggered by a diffusible bacterial product, which is also supported by a similar pattern of melanin production during *Sporothrix* spp. growth in contact with heat-killed *P. agglomerans*. Growth of *P. agglomerans* was similar in the presence or absence of the fungus. However, the growth of *S. brasiliensis* and *S. schenckii* was initially inhibited, but further enhanced when these species were co-cultured with *P. agglomerans*. Moreover, fungi were able to use killed bacteria as both carbon and nitrogen sources for growth. Representational difference analysis identified overexpressed genes related to membrane transport when *S. brasiliensis* was co-cultured with the bacteria. The down-regulation of metabolism-related genes appears to be related to nutrient availability during bacterial exploitation. These findings can lead to a better knowledge on *Sporothrix* ecology and virulence.

Keywords *Pantoea agglomerans* · *Sporothrix brasiliensis* · *Sporothrix schenckii* · Microbial interaction

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Introduction

Sporothrix brasiliensis and *Sporothrix schenckii* are the major agents of sporotrichosis. This subcutaneous mycosis has a broad range of clinical manifestations, from the fixed cutaneous form, where the fungus remains restricted to the implantation site, to the extracutaneous form, with fungal dissemination to several organs and systems, such as bone and central nervous system [1]. Factors that lead to these different clinical manifestations are not well understood, but include inoculum size, depth of traumatic inoculation through skin, host immunity, virulence, and thermotolerance [2].

Infection usually occurs when the fungus penetrates into a susceptible host through a traumatic injury with an environmental material, although more recently zoonotic transmission from naturally infected cats is also reported [1]. *Sporothrix* species have several habitats, but the most common environmental sources where these fungi can be found include rose thorns [3], *Sphagnum* moss [4], hay [5], and soil [6, 7]. The main species isolated from environmental samples is *S. schenckii*; however, *S. brasiliensis* was also identified, in a lesser extent, on soil samples [7, 8].

The microbial survival under environmental conditions is based on different interactions between different bacteria, different fungi, and among bacteria and fungi. These interactions are common in the habitat and can modulate the microbial pathogenesis [9]. In the environment, *Sporothrix* is in an intimate contact with other organisms, from bacteria to small invertebrates. Interactions between pathogenic fungi and bacteria have been studied in the last decade, especially for *Candida albicans* [10–13], *Saccharomyces cerevisiae* [14, 15], and *Cryptococcus neoformans* [16–18], but for *Sporothrix* spp. this subject is poorly addressed. The *Sporothrix* microbial interaction studied so far described that when *S. schenckii* is ingested by *Acanthamoeba castellanii*, a soil-living amoeba, the fungus kills the protozoan and uses it as nutrient for growth [19]. More recently, we have shown that *S. brasiliensis* produces higher amounts of melanin when in contact with some bacteria, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, and *Streptococcus agalactiae* [20].

Pantoea agglomerans, formerly *Enterobacter agglomerans*, is a Gram-negative bacterium associated with plants, soil, arthropods, dust, and air [21]. It can cause opportunistic infections in individuals with an impairment of the immune system after inoculation with plant material [22]. It has been reported the isolation of *P. agglomerans* from *Sphagnum* moss [23], soil [24], and from a subacute synovitis case after a rose thorn injury [25], indicating similar habitats for this bacterium and *Sporothrix* spp. In this study, we report the interaction between these two species, focusing on aspects related to the fungal biology.

Methods

Bacterial Strain

P. agglomerans strain ATCC 27987 was used throughout this study. This strain was maintained in thioglycolate broth (Difco Laboratories, Sparks, MD, USA), and, before experiments, single colonies were obtained through serial dilutions into cystine–lactose–electrolyte-deficient agar plates (Difco Laboratories) at 35 °C.

Fungal Strains

Seventy-one *Sporothrix* strains were used in this study, comprising 10 *S. schenckii* and 61 *S. brasiliensis* strains. Several melanization patterns were observed among these fungal strains, that is, 35 strains are heavily melanized in Sabouraud dextrose agar (SDA) (Difco Laboratories), 28 strains present mild melanin production, and eight strains do not produce melanin in this culture medium, as previously described [26]. The melanin-producing fungal strains *Alternaria brassicicola* ATCC 6650, *Aspergillus niger* ATCC 1004, *Aureobasidium pullulans* ATCC 9348, *C. neoformans* ATCC 32045, and *Cryptococcus gattii* ATCC 56990 were used as control in melanin evaluation experiments.

Sporothrix/*P. agglomerans* Co-cultivation

One-hundred microliters of a suspension in phosphate-buffered saline (PBS) containing approximately 1×10^6 conidia/mL of the *Sporothrix* strains was inoculated on the entire surface of a SDA plate using a Drigalski spatula. Plates were left to dry at room temperature for 15 min. Then, 10 µL of a suspension

in PBS of the *P. agglomerans* strain (1×10^6 cells/mL) was spotted in the center of the plates previously inoculated with the fungus. Controls consisted in SDA plates inoculated with the fungus, as described before, but without bacterium and also an SDA plate inoculated only with *P. agglomerans*. Moreover, the melanin-producing fungi described before were co-incubated with *P. agglomerans* as described for the *Sporothrix* strains. After 7 days of incubation at 30 °C, plates were visually inspected for fungal and bacterial growth, as well as melanin production by the *Sporothrix* strains. Microscopic evaluation of the fungal growth was performed through lactophenol mounts observed under a 1000 × magnification in a Zeiss Axiolab light microscope. After this initial screening, one *S. brasiliensis* and one *S. schenckii* strain with differences in growth and melanization when co-cultured with *P. agglomerans* were selected to be used in further experiments.

Influence of Melanization Inhibitors

The selected *Sporothrix* strains were cultivated in the presence of *P. agglomerans* as described above. Additionally, SDA plates containing tricyclazole (8 mg/L), sulcotrione (16 mg/L), or glyphosate (100 mg/L), all from Sigma-Aldrich, Co., St. Louis, MO, USA, were prepared and inoculated with the *Sporothrix* strains and *P. agglomerans*. After 7 days of incubation at 30 °C, the formation of pigment in plates with melanization inhibitors was compared visually with the pigment in SDA control plates.

Influence of pH

A co-cultivation of *S. brasiliensis* or *S. schenckii* with *P. agglomerans* was performed as described above in SDA plates buffered with 0.164 mol/L 3-(N-morpholino)propanesulfonic acid (MOPS) (InLab, São Paulo, Brazil) or PBS at pH 7.0. Melanization in these plates was compared with that in the SDA control plates after 7 days of incubation at 30 °C. Moreover, the melanin production in the presence of bacterium in SDA without buffer was compared with the data about the melanization of these strains in acid and neutral pH, as described previously by our group [27], by using SDA plates with pH 4.5 and 7.0 incubated at 30 °C during 7 days.

Influence of Temperature

The *Sporothrix* strains and *P. agglomerans* were co-cultivated as described before and incubated at 36 °C during 7 days. Production of melanin in this condition was compared with control cultures incubated at 30 °C.

Growth Curves

An inoculum of 1×10^8 *Sporothrix* conidia was co-cultivated with 1×10^8 *P. agglomerans* cells in Erlenmeyer flasks containing 100 mL of Sabouraud dextrose broth (SDB) (Difco Laboratories). Controls consisted in pure cultures of the *S. brasiliensis*, *S. schenckii*, and *P. agglomerans* strains in the same culture medium and under the same incubation conditions. After homogenization, an aliquot of 1 mL was obtained and tenfold serial dilutions were prepared in PBS. Plates containing brain heart infusion (Difco Laboratories) and SDA with 400 mg/L chloramphenicol were used for bacterial and fungal colony forming unity per milliliter (CFU/mL) counts, respectively. The inoculated Erlenmeyer flasks were incubated in a rotary shaker (150 rpm) for 7 days at 30 °C. The CFU/mL counts for fungi and bacteria were determined daily throughout the incubation period and used for the construction of growth curves.

Melanin Ghosts

The *Sporothrix* strains grown in SDB in the presence or absence of *P. agglomerans*, as described before, were harvested by centrifugation and filtered through a nylon membrane disk filter with 20 µm pore size to remove bacteria, as described [28]. The fungal biomass was submitted to enzymatic, denaturant, and hot acid treatments to obtain fungal melanin particles, also known as "melanin ghosts," as previously described [27].

Conventional Scanning Electron Microscopy

Melanin ghosts were adhered onto coverslips coated with 0.01% poly-L-lysine (Sigma-Aldrich), for 20 min and dehydrated in a series of freshly made solutions of graded ethanol (30, 50, and 70%, for 5 min/step, then 95% and twice 100%, for 10 min/step). Samples were

then subjected to critical point drying (EM CPD 300, Leica) immediately after dehydration, mounted on metallic stubs, coated with a 15–20 nm gold layer (Balzers Union FL-9496, Balzers), and visualized in a scanning electron microscope (FEI Quanta 250), operating at 10–20 kV.

Growth Assay in the Presence of Conditioned Bacterial Medium

A conditioned bacterial medium was prepared by growing 1×10^6 cells/mL of *P. agglomerans* in 100 mL SDB for 5 days at 30 °C. After incubation, bacterial cells (approximately 1×10^9 cells/mL) were removed by centrifugation (10,000 g) and filtering through a nitrocellulose membrane with a 0.22 µm pore diameter size. The supernatant was concentrated 50 times, that is, to a final volume of 2 mL. This concentrated supernatant was mixed with 98 mL of freshly prepared SDA, and this conditioned medium (c-SDA) was inoculated with 10 µL of a suspension containing 1×10^8 *Sporothrix* conidia/mL. Growth and pigment production were evaluated after 7 days of incubation at 30 °C.

Influence of Bacterial Quorum-Sensing Molecules

Conidia of the *Sporothrix* strains were harvested from 7-day-old cultures in order to prepare suspensions containing 1×10^8 conidia/mL, and serial tenfold dilutions were performed. Aliquots of 10 µL of the 10^{-1}–10^{-3} dilutions were spotted in SDA plates containing 25 µg/mL of one of the following quorum-sensing molecules diluted in dimethyl sulfoxide (DMSO): *N*-butanoyl-L-homoserine lactone (BHL), 2,4-dihydroxyquinoline (DHQ), *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL), *N*-hexanoyl-L-homoserine lactone (HHL), or 3,4-dihydroxy-2-heptylquinoline (PQS), all from Sigma-Aldrich. The final DMSO concentration in these plates was 0.2%, and a control SDA plate containing 0.2% DMSO was also used to inoculate the serial dilutions of *Sporothrix* conidia. Final conidia amount inoculated on the spots was 1×10^5, 1×10^4, and 1×10^3, respectively. Plates were incubated at 30 °C in the dark and observed daily during 7 days, checking for changes in growth or melanization of the strains.

Growth Assay Using *P. agglomerans* as Nutrient

The bacterial cells were harvested from 5-day-old pure cultures in SDB and killed by autoclavation at 121 °C, during 30 min. The killed bacterial cells were washed three times in PBS and dried at 65 °C overnight. To check whether *S. brasiliensis* and *S. schenckii* would be able to use *P. agglomerans* as carbon and nitrogen sources for growth, culture media containing 0.5 g of killed bacteria, 1.5 g agar, and respectively 0.67 g yeast nitrogen base (YNB) or 1.17 g yeast carbon base (YCB), both from Difco Laboratories, in 100 mL distilled water were prepared. Additionally, media containing only 0.5 g of killed bacteria and 1.5 g agar (Kasvi, São José dos Pinhais, Brazil) in 100 mL distilled water were used to check if the fungi also would be able to consume the bacterium as both carbon and nitrogen sources, concomitantly. Positive controls consisted in YNB plates supplemented with 0.5% dextrose and negative controls consisted in YNB plates without carbohydrate addition. An inoculum of 1×10^6 *Sporothrix* conidia/mL was prepared in PBS and inoculated in triplicate using a toothpick-wound procedure in the above-mentioned media. After 14 days of incubation at 30 °C, the diameter of the colonies was measured using a millimeter ruler. This experiment was performed in triplicate in three different days, resulting in nine measurements for each strain in each culture medium.

Representational Difference Analysis

This study was conducted as previously described [29] with minor modifications. In brief, the *S. brasiliensis* strain was grown at 30 °C in SDB medium and 1×10^6 conidia transferred to two different cultures: a tester culture, in 50 mL of SDB where 1×10^6 *P. agglomerans* was also inoculated, and a driver culture, in 50 mL of SDB without the bacterium. Both cultures were incubated at 30 °C under 150 rpm rotary shaking for 7 days. Total RNA from *S. brasiliensis* was extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, USA). The first-strand DNA synthesis was made with reverse transcriptase (RT Superscript III, Invitrogen Life Technologies) from 1.0 µg total RNA. This strand was used as template for the second-strand DNA synthesis using the SMART PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA). The cDNA fragments were digested with *Sau3*AI

restriction enzyme (Promega, Madison, WI) to construct cDNA libraries of the fungus grown in the two above conditions. The resulting products were purified using the GFX kit (GE Healthcare, Chalfont St. Giles, UK). To generate subtraction products, tester and driver cDNAs were mixed and hybridization occurred at 67 °C for 18 h. Two subtraction rounds were performed, using tester/driver cDNA ratios of 1/10 and 1/100, respectively. After the second subtraction, the amplified cDNAs were cloned in pGEM-T Easy Vector (Promega, Madison, EUA), which was used to transform the *E. coli* XL1-blue strain. Automated sequencing was performed using the MegaBACE 1000 DNA sequencer (GE Healthcare). Sequences were analyzed as described before [29]. The database sequence matches were considered significant at e-values less than 10^{-5} with a sequence coverage cut of 40%.

Statistical Analysis

Descriptive statistical analyses were performed with the GraphPad Prism 6 software. Fisher exact test was employed to a contingency table to correlate melanization in the presence of bacterium and at acid pH. The Mann–Whitney test was used to compare fungal colony diameters under different nutritional conditions. A P value less than 0.05 was considered statistically significant.

Results

P. agglomerans Induces Melanization in *Sporothrix* spp.

Differences in the bacterial growth during the co-cultivation of the different *Sporothrix* strains with *P. agglomerans* in a solid substrate, that is, SDA plates, were not observed when comparing plates with or without fungi. Both *S. brasiliensis* and *S. schenckii* were able to produce melanin when in contact with the bacterium (Fig. 1). However, 50 *Sporothrix* strains (81.97%), 42 *S. brasiliensis* and 8 *S. schenckii*, presented melanized cells around *P. agglomerans* colonies. It was observed that 42 of them (35 *S. brasiliensis* and 7 *S. schenckii*) were heavily melanized, whereas eight (seven *S. brasiliensis* and one *S. schenckii*) presented a slight enhancement of melanin production. Interestingly, one *S. schenckii* strain previously described as a non-melanin producer [26] was found to produce slight amounts of melanin around *P. agglomerans*. Significant differences in fungal melanization around bacterial colonies at 30 °C and 36 °C were not observed, except for two *S. brasiliensis* strains that produced smaller and lighter melanin halos around *P. agglomerans*. When it was checked whether a similar interaction occurs between *P. agglomerans* and other fungi, it was found that this bacterium does not influence growth or melanization of *A. niger* and *A. pullulans*. Moreover, neither *C. neoformans* nor *C. gattii* produced melanin around the bacterium. On the other hand, it was observed that, contrary to the *Sporothrix* behavior in the presence of *P. agglomerans*, this bacterium was able to inhibit both growth and melanization of *A. brassicicola*, which was unable to grow in contact and near the bacterial colony in SDA plates.

Tricyclazole Inhibits *P. agglomerans*-Driven Melanization of *Sporothrix*

Of the melanization inhibitors included in this work, only the DHN-melanin inhibitor tricyclazole was able to hinder pigment formation around *P. agglomerans* colonies in all tested *Sporothrix* strains (Fig. 2a). Melanization of the *Sporothrix* colonies around *P. agglomerans* colonies in the presence of glyphosate (Fig. 2b) or sulcotrione (Fig. 2c), eumelanin and pyomelanin inhibitors, respectively, was similar to the controls without inhibitors.

The pH is Important During *Sporothrix*/*P. agglomerans* Interaction

Since the pH of pure *P. agglomerans* cultures becomes acid, that is, around 5.0, after 5 days of incubation, the interaction of these two organisms was studied in buffered media. It was observed a lack in fungal melanization around bacterial colonies in the media buffered at pH 7.0, even after a prolonged 10-day incubation time, independent of the buffer or strain used (Fig. 3). The melanization of pure cultures of *Sporothrix* strains at pH 4.5 and 7.0 revealed that eight strains (11.27%) were unable to produce melanin in both conditions (three *S. brasiliensis* and five *S. schenckii*), 29 (40.85%) presented the same melanization profile at both pHs (26 *S. brasiliensis* and three *S.*

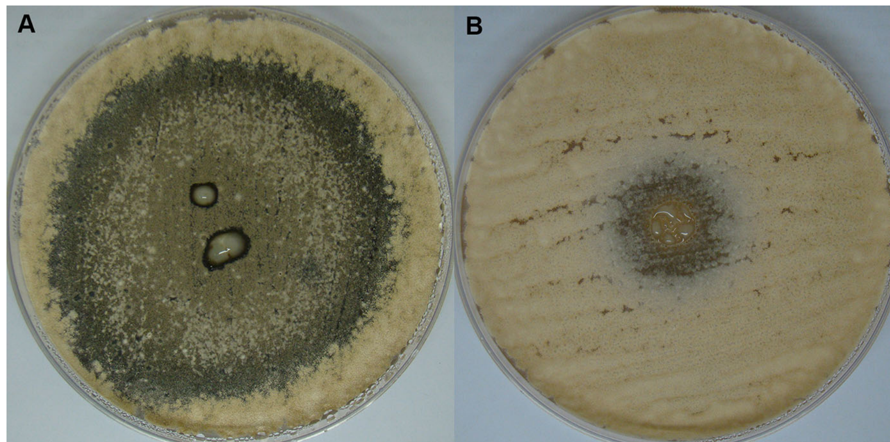

Fig. 1 Co-cultures of *Pantoea agglomerans* with *Sporothrix brasiliensis* (**a**) or *Sporothrix schenckii* (**b**) after 7 days of incubation at 30 °C

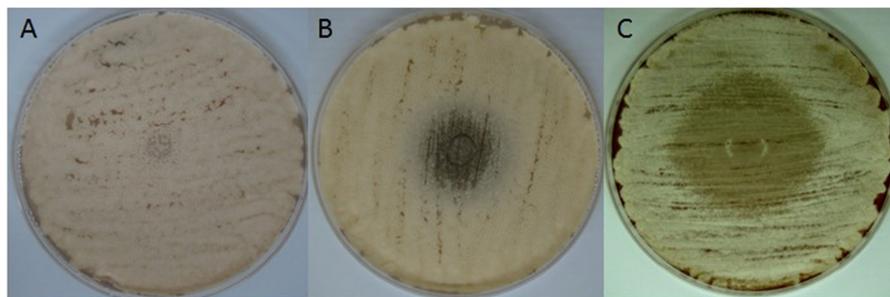

Fig. 2 Co-cultivation in the presence of tricyclazole (**a**), a DHN-melanin inhibitor; glyphosate (**b**), an eumelanin inhibitor; and sulcotrione (**c**), a pyomelanin inhibitor

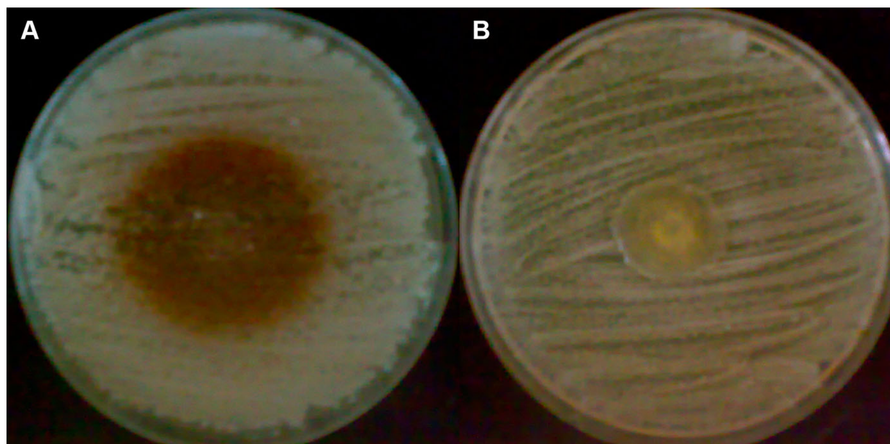

Fig. 3 Co-cultures of *P. agglomerans* with *S. brasiliensis* in Sabouraud dextrose agar (**a**) and Sabouraud dextrose agar buffered at pH 7.0 (**b**) after 7 days of incubation at 30 °C

schenckii), 14 (19.72%) produced more melanin at neutral pH (13 *S. brasiliensis* and 1 *S. schenckii*), and 20 (28.17%) were more melanized at acid pH (19 *S. brasiliensis* and one *S. schenckii*). The comparison of melanization around *P. agglomerans* colonies with acid pH showed that 39 strains presented similar

behaviors in both conditions. However, 30 strains had increased melanin production around *P. agglomerans* but not in acid SDA plates, and two strains produced more melanin at acid pH but not around bacterial colonies. Fisher exact test revealed that the association between these two melanization conditions is statistically significant ($P = 0.0121$).

P. agglomerans Modulates *Sporothrix* Melanization Through Secreted Compounds

After 7 days of incubation at 30 °C, the *S. brasiliensis* and *S. schenckii* colonies in SDA were white (Fig. 4a). However, when cultured in medium conditioned with a filtered supernatant of *P. agglomerans* cultures (c-SDA), both tested *Sporothrix* spp. strains presented significant melanization (Fig. 4b). Microscopically, hyaline conidia were observed in the SDA cultures of *Sporothrix* spp. (Fig. 4c), whereas many dematiaceous conidia were observed in *Sporothrix* spp. cultures performed in c-SDA (Fig. 4d). In addition, of the bacterial quorum-sensing molecules tested, it was observed that HHL and PQS enhanced *S. brasiliensis* melanization after 4 days of growth, but no effect on *S. schenckii* was observed for all tested molecules (Fig. 4e).

Fungal Melanin Ghosts can be Isolated After *P. agglomerans/Sporothrix* Co-cultivation

No melanin particles were isolated from pure bacterial colonies. However, particles retaining the shape and size of *Sporothrix* conidia were obtained from both *Sporothrix* species in the presence or absence of the bacterium. Scanning electron microscopy showed similar topology and size of the melanin particles generated in the presence or absence of *P. agglomerans* (Fig. 5).

P. agglomerans Modulates *Sporothrix* Growth

It was observed that the presence of *Sporothrix* in the culture medium does not affect *P. agglomerans* growth. However, differences in fungal growth curves in the presence or absence of the bacterium were observed. In the absence of *P. agglomerans*, both *S. brasiliensis* and *S. schenckii* reached log phase after 2 days of growth. However, in the presence of the bacterium, *Sporothrix* spp. remained in lag phase after this time. After death phase of bacterial growth, *Sporothrix* spp. entered the exponential phase after 3 days, and after 4 days, both species went into stationary phase, with more viable cells than the control culture without the bacterium (Fig. 6). A microscopic analysis of cultures grown in broth media after 4 days was similar to cultures in solid media, that is, there was a predominance of dematiaceous or hyaline conidia in the presence or absence of the bacterium, respectively.

Sporothrix spp. can Use *P. agglomerans* as Nutrient for Growth

As depicted in Fig. 7, neither *S. brasiliensis* nor *S. schenckii* were able to grow significantly when incubated in YNB medium without carbohydrate. Incubation of the fungi in YNB or YCB supplemented with heat-killed bacterial cells resulted in colonies of similar size to those grown in YNB supplemented with glucose. Moreover, both species were able to grow using *P. agglomerans* as the sole nutrient for growth, although colony diameters in this culture condition were smaller than controls ($P < 0.05$).

P. agglomerans Modulates *Sporothrix* Gene Expression

A total of 15 different contigs presenting e-values lower than 10^{-5} after Blast X analysis were obtained using RDA analysis. From these, eight contigs were down-regulated and seven were up-regulated when *S. brasiliensis* was grown in the presence of *P. agglomerans*. The distribution of differentially expressed genes in biological function groups was assessed. It was observed that down-regulated genes participated in cellular processes related to metabolism, transcription, protein synthesis, binding function, and stress response. Up-regulated genes were found to participate in transcription regulation, protein fate, regulation of protein activity, cellular transport, transport facilities, and transport routes (Table 1).

Discussion

Microbial survival in the environment is based in different interactions between bacterial species, fungal species, and between fungi and bacteria, and microbial

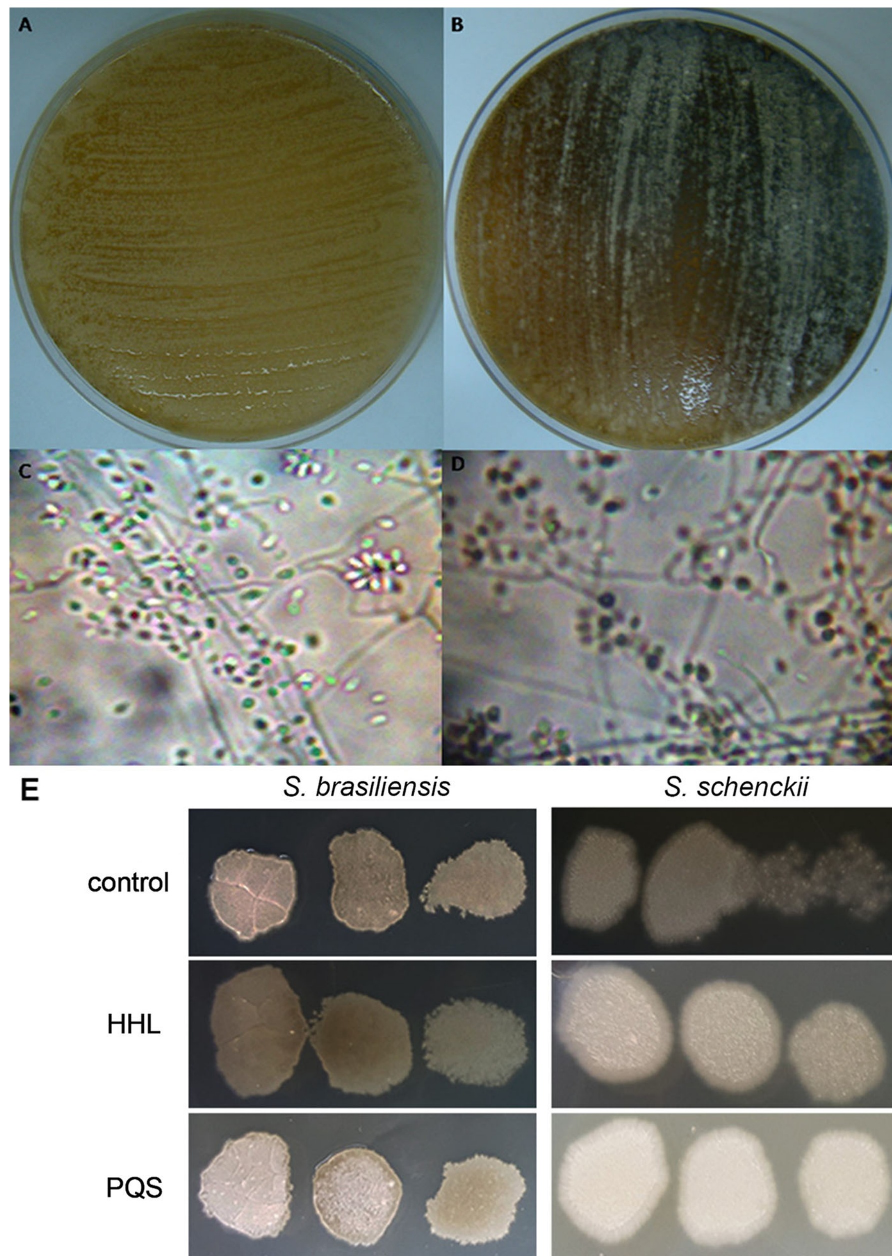

Fig. 4 Macroscopic aspect of a *S. brasiliensis* culture in Sabouraud dextrose agar (**a**) and Sabouraud dextrose agar conditioned with the supernatant of a *P. agglomerans* culture (**b**). Microscopic aspect of a *S. brasiliensis* culture in Sabouraud dextrose agar (**c**) and Sabouraud dextrose agar conditioned with the supernatant of a *P. agglomerans* culture (**d**) after 4 days of incubation. Effect of the bacterial quorum-sensing molecules (**e**) *N*-hexanoyl-L-homoserine lactone (HHL) and 3,4-dihydroxy-2-heptylquinoline (PQS) in three *S. brasiliensis* or *S. schenckii* strains

pathogenesis can also be modulated by these interactions [9]. Melanin production by *Sporothrix* species is quite complex and the occurrence of this process in saprophytism or during parasitism, conditions in which the fungus is interacting with other organisms, is certainly modulated by different conditions than those studied in the laboratory [20]. As an example of the importance of fungal/bacterial interaction in the

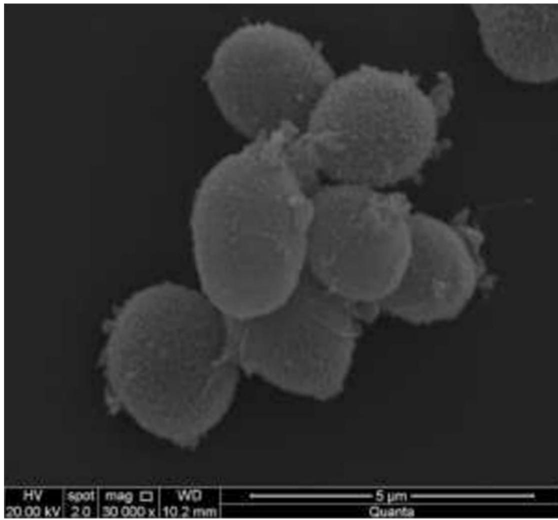

Fig. 5 Scanning electron microscopy of a *S. brasiliensis* strain co-cultivated with *P. agglomerans* after denaturant and hot acid treatment. Similar *S. schenckii* co-cultures yielded similar melanin particles. Bar: 5 µm

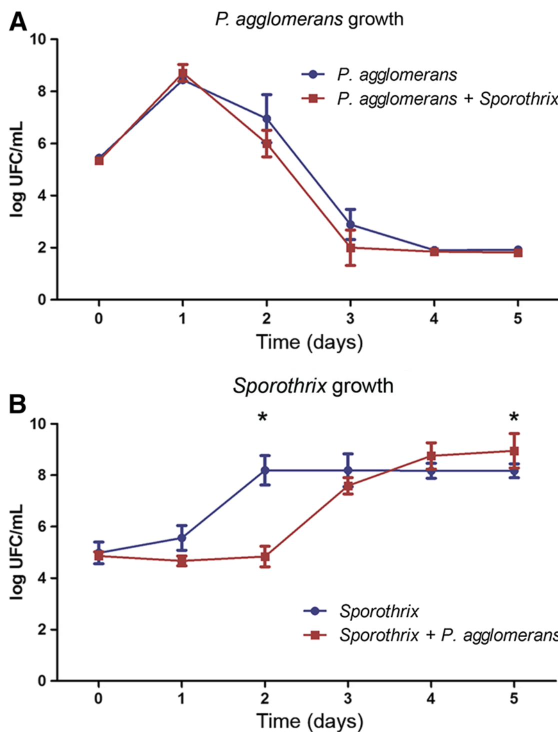

Fig. 6 Bacterial (a) and fungal (b) growth curves of a representative *S. brasiliensis*/*P. agglomerans* co-cultivation in Sabouraud dextrose broth. $*P < 0.05$

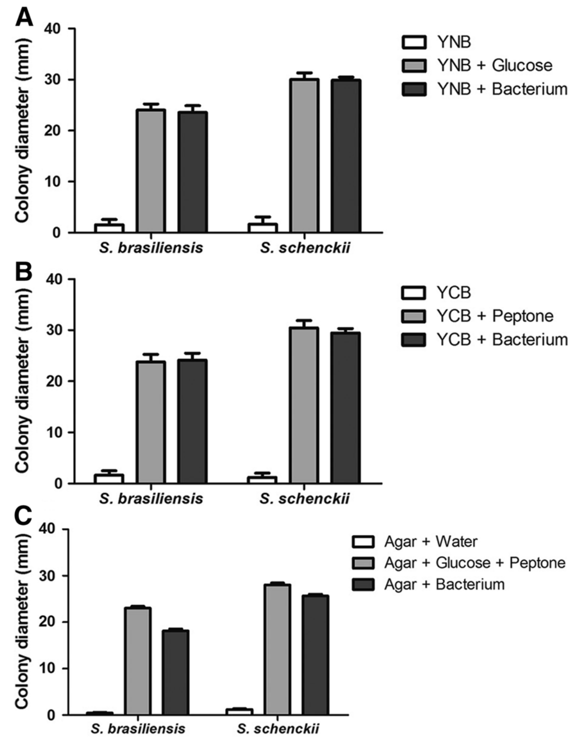

Fig. 7 Use of *P. agglomerans* as a carbon source by *S. brasiliensis* and *S. schenckii* (**a**). Use of *P. agglomerans* as a nitrogen source by *S. brasiliensis* and *S. schenckii* (**b**). Use of *P. agglomerans* as a sole nutrient for *S. brasiliensis* and *S. schenckii* growth (**c**). In all experiments, unsupplemented media were used as negative controls and supplemented media were used as positive growth controls

fungal melanogenesis process, the interaction between *C. neoformans* and *Klebsiella aerogenes* leads to yeast melanization through the bacterial supply of dopamine as a melanin precursor for the fungus [16]. In this study, we have shown another fungal/bacterial interaction with potential to modulate melanin biosynthesis by pathogenic fungi, reinforcing the theory that the *Sporothrix* virulence factors are modulated by environmental stressors [19, 30].

Although *P. agglomerans* is able to induce melanization in *S. brasiliensis* and *S. schenckii*, the mechanisms leading to this process are different to that previously observed for *C. neoformans* and *K. aerogenes* [16], since neither *C. neoformans* nor *C. gattii* were able to produce melanin in contact with *P. agglomerans*, and because DHN-melanin is likely to be produced during *Sporothrix* spp./*P. agglomerans* interaction. The production of this type of melanin is supported by the inhibition of pigment production by

Table 1 Up- and down-regulated genes of *Sporothrix brasiliensis* mycelial cells in the presence of *Pantoea agglomerans* as detected by representational difference analysis

Functional category	Gene product (accession number)	e-value	Regulation
Amino acid metabolism	Glutamine synthetase (KIH94590)	1e−06	Down
Carbohydrate metabolism	Cysteine desulfurase (KIH88569)	8e−19	Down
Transcription	GATA transcription factor, white collar-2 protein homolog (KIH89252)	1e−61	Down
	Siderophore transcription factor (KIH89362)	5e−09	Up
	Histone deacetylase (KIH91941)	1e−59	Up
Protein synthesis	Seryl-tRNA synthetase (KIH88641)	2e−41	Down
Protein Fate	Ubiquitin carboxyl-terminal hydrolase 19 homolog (KIH90660)	4e−08	Up
Binding function	Actin-interacting protein (KIH90749)	9e−44	Down
Regulation of protein activity	Guanine nucleotide exchange factor (KIH87120)	3e−22	Up
Cellular transport, transport facilities, and transport routes	Major facilitator superfamily transporter (KIH91962)	5e−55	Up
	Major facilitator superfamily transporter homolog (KIH94664)	7e−68	Up
Stress response	Heat shock protein 70 (KIH91446)	7e−26	Down
	Chaperonin GroEL (KIH94969)	1e−90	Down
Unclassified proteins	Hypothetical protein (KIH91906)	7e−10	Down
	Hypothetical protein (KIH92742)	2e−14	Up

tricyclazole, a potent DHN-melanin inhibitor [31], and by the fact that only conidial melanin ghosts were generated from *Sporothrix* cells grown in the presence of *P. agglomerans*, that is, hyphal forms are not melanized, which would have occurred if DOPA melanin was produced [27].

Our group has previously demonstrated that the pH modulates melanization by species of *Sporothrix* [27]. Therefore, an initial hypothesis for the enhanced melanin production around bacterial colonies was that the acidification of the environment caused during *P. agglomerans* growth would lead to *Sporothrix* melanization. The results herein presented support partially this hypothesis, because although a statistically significant association has been found for melanization around bacterial colonies and melanization in acid pH, there were strains unable to enhance melanin synthesis at pH 4.5 but that melanize in contact with *P. agglomerans*. Moreover, cell-free secreted bacterial compounds were able to enhance melanin synthesis in the same way that was observed when the fungus has grown with live bacterial cells, and quorum-sensing molecules, particularly HHL and PQS, were able to trigger melanin production only by *S. brasiliensis*. These results show that the process leading to *Sporothrix* melanization in the presence of *P. agglomerans* is multifactorial and species-dependent.

Quorum-sensing molecules are usually involved in fungal/bacterial interactions [9]. For instance, small diffusible extracellular molecules of *P. aeruginosa* have an inhibitory action against *Aspergillus fumigatus* [32]. It has been demonstrated that *P. agglomerans* produce homoserine lactone quorum-sensing signals, especially BHL and HHL in major and minor concentrations, respectively [33]. These compounds have been associated with *P. agglomerans* virulence for plants and our results suggest that at least HHL is also associated with a fungal/bacterial interaction, especially in a pathogenic fungus. Since clinical and plant strains of *P. agglomerans* are phylogenetically indistinguishable [34], it is possible that this quorum-sensing molecule acts not only in the environment, but also in mixed infections caused by these species or other bacteria that can produce HHL. Further studies are necessary to confirm this hypothesis. The other quorum-sensing signal with influence on *Sporothrix* melanization, PQS, is usually produced by *P.*

aeruginosa [35], and we were not able to find reports of the production of this molecule by *P. agglomerans*. These results suggest that *Sporothrix* melanization in the presence of other bacteria [20] may also be driven by quorum-sensing molecules rather than HHL.

The interaction between *Sporothrix* spp. and *P. agglomerans* seems to involve complex regulatory mechanisms. It was observed that both *Sporothrix* species tested are unable to modulate bacterial development; however, *P. agglomerans* alters fungal growth. During the interaction between *S. cerevisiae* and *Pseudomonas putida*, the fungal metabolism of glucose prevents a rapid acidification of the pH, which leads to an enhanced bacterial stationary phase of growth [36]. Moreover, this same fungus is able to modulate the growth of another bacterium, *Lactobacillus delbrueckii* [37]. Together with the results herein presented, it is demonstrated that fungal influence on the bacterial growth is species-dependent. The growth of *Sporothrix* spp. in the presence of *P. agglomerans* occurs only after bacterial death, when the amount of nutrients in the culture medium was reduced by the bacterium. It has been demonstrated that *S. schenckii* can use *A. castellanii* as a nutrient for growth [19], which also happens with *P. agglomerans*. In fact, the fungus can use dead *P. agglomerans* cells as both carbon and nitrogen sources for growth, being able to grow in nutrient-deficient environments. It has been demonstrated that some bacteria, particularly those from the genus *Collimonas* are able to exploit fungi as nutrient sources, in a process named mycophagy [38]. Our results point to an inverse interaction between *Sporothrix* spp. and *P. agglomerans*.

It is interesting to note that most conidia produced by the two *Sporothrix* species tested after *P. agglomerans* death are dematiaceous. In a study of the interaction between *Aspergillus nidulans* and soil actinomycetes, it has been demonstrated that the polyketide synthase-encoding gene is up-regulated when the fungus is maintained in contact with these bacteria [39]. A similar process should happen with *Sporothrix* spp., and therefore, the interaction herein studied affects *Sporothrix* virulence by increasing the number of fungal infectious particles and by an enhanced melanization in them.

The data generated through the RDA study allowed the identification of *S. brasiliensis* genes that are impacted by the presence of *P. agglomerans* in the same environment. Unfortunately, we were unable to identify genes directly related to melanin synthesis pathways in our study. This might be partially due to the paucity of well-characterized proteins of the pathogenic species of *Sporothrix*. In fact, genome sequences of *S. schenckii* [40] and *S. brasiliensis* [41] are very new and to the best of our knowledge there are no specific reports about characterization of enzymes or gene sequences related to melanization in the two pathogenic species of *Sporothrix* included in this study. However, some of the identified genes have a role in fungal virulence [42, 43].

Interestingly, three genes involved in transcription process were differentially regulated. A GATA transcription factor with 100% homology to the white collar 2 (wc2) protein of *S. schenckii* was found to be down-regulated, while a siderophore transcription factor and a histone deacetylase were up-regulated. The wc2 protein is a GATA transcription factor that binds the white collar 1 protein forming a complex involved in blue light sensing [44]. It has been described that for fungi that produce more conidia in the presence of light, such as *Fusarium graminearum* [45] or *Trichoderma atroviride* [46], wc2 enhances conidiogenesis, while for fungi that produce more conidia in the dark, such as *Alternaria alternata* [47], wc2 impairs conidiogenesis. Since *Sporothrix* spp. can produce large amounts of conidia in the dark [48, 49], the down-regulation of wc2 could be an explanation for the enhanced *Sporothrix* growth in the presence of *P. agglomerans*. Histone deacetylases remove acetyl groups from histones, participating in transcriptional regulation and, in fungi, they are related to the expression of virulence-related genes [42, 50, 51]. Moreover, a histone deacetylase induces conidiation in *T. atroviride* [52]. Taken together, the down-regulation of the wc2-related GATA transcription factor and the up-regulation of a histone deacetylase provide a molecular explanation for the luxuriant growth of *S. brasiliensis* when grown in contact with *P. agglomerans*. The up-regulation of a *S. brasiliensis* siderophore transcription factor in the presence of *P. agglomerans* should be a reflex of fungal iron uptake mechanisms in the context of a competition between these two organisms for this essential metal.

The down-regulation of two *S. brasiliensis* metabolism-related genes, cysteine desulfurase and glutamine synthetase, must be a reflex of the carbon and nitrogen sources available for the fungus during the

exploit of bacterium for growth. Cysteine desulfurase is an enzyme that cleaves sulfur from cysteine to form alanine [53]. For instance, a recent study has pointed that cysteine has deleterious effects for some bacteria, including *Pantoea ananatis*, which produces a cysteine desulfhydrase to degrade cysteine and possesses cysteine efflux bombs that guarantee very low levels of this molecule in the bacterial cell [54]. Glutamine synthetase catalyzes the condensation of glutamate and ammonium, at the cost of ATP hydrolysis, to produce glutamine [55]. It has been demonstrated that *P. agglomerans* maintains a high level of glutamine within the cells, which is necessary to produce compounds involved in competiveness with other organisms living in the same habitat [56].

Two major facilitator superfamily (MFS) genes of *S. brasiliensis* were up-regulated during fungal/bacterial interaction. These transporters are necessary for the traffic of a wide range of molecules across the plasma membrane [57]. In *Penicillium funiculosum*, the MFS transporters are related to acidic conditions [58], an environmental condition that *S. brasiliensis* must face during *P. agglomerans* interaction. Also, they confer resistance to toxic compounds or molecules with antifungal properties produced by other organisms living in the same habitat [59, 60]. Therefore, the *S. brasiliensis* MFS transporters must have a role in the protection against *P. agglomerans*-driven acidification of the medium and against potential toxic molecules that are produced by this bacterium. Furthermore, the overexpression of these genes may impact *S. brasiliensis* resistance against antifungal drugs. MFS transporters are usually associated with drug resistance through the extracellular transport of the antifungal molecules by the efflux pumps [61]. MFS transporters are associated with resistance to voriconazole [62] and fluconazole [63], two antifungal drugs that are ineffective against *S. brasiliensis* [64–66].

Surprisingly, two different stress response proteins, chaperonin GroEL and heat shock protein (HSP) 70, were down-regulated in the context of fungal/bacterial co-cultivation. In eukaryotes, chaperonin GroEL is a highly conserved homolog of HSP60 [67]. These proteins are chaperones typically linked to the response to cellular stress, having their expression increased during exposure to heat, cold, ultraviolet light, and compounds with antifungal properties [29, 68]. This result suggests that interaction with bacteria is not a stressful condition for *S. brasiliensis*, probably because it is adapted to live together with other microorganisms in the environment.

The finding that *S. brasiliensis*, *S. schenckii*, and *P. agglomerans* can be isolated from soil samples [8, 24] supports the existence of a real interaction between them. The data demonstrate that this interaction induces the expression of virulence-related phenotypes, including DHN-melanin, the constitutive melanin type produced by *Sporothrix* spp. These support the theory that fungal virulence evolved from inter-microbial interactions during environmental growth [69].

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Research Involving Human Participants and/or Animals This article does not contain any studies with human participants and/or animals performed by any of the authors.

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